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14. ABSTRACT

Estrogen plays a significant role in the progression of breast cancer, leading to increased growth of breast tumors. Until 2005, the classical estrogen receptor, ERα, was believed to modulate the entirety of E2-dependent breast cancer growth. In 2005, our lab demonstrated that estrogen binds to and activates the orphan G protein-coupled receptor, GPR30, which has been renamed G Protein Coupled Estrogen Receptor (GPER). Since the discovery of GPR30 as a novel estrogen receptor, it has been established that activation of GPR30 enhances proliferation in many cell types. However, the *in vivo* effects of GPR30 in breast cancer have not been established. Our preliminary results in a mouse model of breast tumorigenesis demonstrate that inhibition of GPER reduces estrogen-mediated tumor growth.

15. SUBJECT TERMS
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Introduction
The steroid hormone, estrogen (17β-estradiol or E2), is involved in numerous and varied physiological processes. It is best characterized for its roles in female reproduction and breast development [1]. Until recently, all E2-dependent effects were thought to be propagated exclusively through the classical estrogen receptors, ERα and ERβ. However, in double ERα/β knockout mice, select E2-dependent effects remain, suggesting the existence of additional E2 receptors [2, 3]. In 2000, an orphan G protein-coupled receptor (GPCR), GPR30, was shown to elicit E2-dependent signaling in SKBr3 cells, which lack both classical E2 receptors [4]. In 2005, our group and another group led by Filardo and Thomas independently demonstrated that E2 bound to GPR30, which has since been renamed G protein-coupled estrogen receptor (GPER) [5, 6].

E2 plays a central role in the progression of breast cancer (BrCa), and enhances the proliferation, migration, and invasion of breast tumor cells *in vitro* and *in vivo* [7, 8]. Inhibiting E2 signaling through a variety of methods in women with BrCa increases long-term survival [9, 10]. One of the most commonly prescribed adjuvant treatments for women with BrCa is tamoxifen, a selective estrogen receptor modulator (SERM) that acts as an antagonist for ERα in the breast, inhibiting tumor growth. Treatment with tamoxifen for 5 years after remission decreases mortality by 31%. However, 25% of women do not respond to tamoxifen or become resistant during treatment [9]. While tamoxifen is a successful therapy, BrCa is still the 2nd most common cause of cancer-related death in women in the US [11], implying that inhibition of E2 signaling solely through ERα is incomplete and other E2 receptors may be involved in the progression of BrCa. Understanding E2 signaling more completely may aid in the treatment of BrCa patients in whom inhibiting ERα is an unsuccessful therapy.

Although GPR30 is an E2 receptor, its role in BrCa is largely unknown. E2 stimulation of GPR30 activates the mitogen activated protein kinase (MAPK) cascade as well as phosphoinositide 3-kinase (PI3K), suggesting a role for GPR30 in proliferation and cell survival in BrCa [4, 12, 13]. Additionally, GPR30 is responsible for E2-dependent proliferation in select BrCa cells *in vitro* [14]. A retrospective study of 361 breast cancer patients found GPR30 expression correlated with an increased size of the primary tumor and the occurrence of distant metastasis [15]. However, in a similar study of inflammatory BrCa patients, expression of GPR30 was not correlated with overall survival or disease free survival, although it was expressed in the majority of samples (67%) [16]. Although GPR30 is able to increase proliferation *in vitro*, the role of GPR30 *in vivo,* where tumor cells exist in a complex microenvironment, remains unclear.

Body
The original proposal for this fellowship presented three specific aims, which included:
Aim 1: GPR30 contributes to estrogen-dependent proliferation.
Aim 2: GPR30 contributes to anti-estrogen resistance in breast cancer.
Aim 3. Does GPR30 expression enhance lymph node and distant metastasis and predict patient outcome?

While the first aim of the proposal has not been changed, aim2 and aim3 have been altered. In **aim2** we proposed to investigate GPR30-dependent anti-estrogen resistance by looking specifically at ERα phosphorylation. However, we have been unable to detect GPR30-dependent ERα phosphorylation in our cells lines. In addition, an independent group who studies GPR30 is also unable to detect GPR30-dependent ERα phosphorylation (personal communication).

The specific objectives of **aim 3** were to measure the expression of GPR30 in matched tissue samples from primary breast tumors and axillary nodal metastases (and where available, also distant metastatic sites) and to correlate GPR30 expression with known clinico-pathologic parameters that predict for patient outcome in breast cancer and patient responsiveness to endocrine therapy. However, a power analysis was performed to determine the number of patient samples needed to obtain significant data, and it was predicted that a large number of patients would be required. We believe that in light of previous publications [15], this data would advance knowledge about the role of GPR30 in breast cancer incrementally. Therefore, we did not believe this aim would be beneficial to my training.

We are currently investigating the effects of GPR30 on the MMTV-PyMT (PyMT$^{+/+}$) mouse model of breast carcinogenesis. Using a mouse model of breast cancer will allow us to examine the role of GPR30 in tumor development and progression in a complex environment. PyMT$^{+/+}$ mice spontaneously develop mammary tumors resulting from the expression of the polyoma middle T antigen (PyMT) specifically in the mammary gland [17]. We have modified aim 2 and aim 3 to reflect the changes in my research focus. Thus, **Aim 2** is to analyze tumor growth, grade, and receptor status of GPR30 knockout PyMT$^{+/+}$ mice (GPR$^{-/-}$PyMT$^{+/+}$) compared to GPR30 wild type PyMT$^{+/+}$ mice (GPR30$^{+/+}$PyMT$^{+/+}$) mice as well as PyMT$^{+/+}$ mice treated with a GPR30-selective agonist and antagonist. **Aim 3** is to determine the extent of distant metastasis in GPR$^{-/-}$ PyMT$^{+/+}$ mice and treated PyMT$^{+/+}$ mice.

Aim 1: The first aim of this proposal is to determine if GPR30 contributes to estrogen-dependent proliferation, specifically in breast cancer cell lines.

To determine if GPR30 contributes to estrogen-dependent proliferation, we utilized a human cell line, SUM149, which was isolated from the pleural effusion of a patient with advanced inflammatory breast cancer (IBC). SUM149 cells are ERα and ERβ negative, overexpress the epidermal growth factor receptor (EGFR), and we have determined by immunofluorescent antibody staining that they are GPR30 positive. We also used a PyMT cell line derived from the tumor of a PyMT$^{+/+}$ mouse. The receptor status of these cells has not been determined. We treated the cells with E2 and the GPR30-selective agonist, G-1, and determined the cell number after 4 days by staining the cells with crystal violet. SUM149 cell number increased in a dose-dependent manner when stimulated with G-1 and E2. PyMT cells had a slight, but significant increase in cell number when treated with G-1. Although crystal violet staining is not a direct measurement of proliferation, stimulation of GPR30 in both SUM149 and PyMT cells increased cell number after 4 days, suggesting an increase in proliferation.

Aim 2: Aim 2 proposes to analyze the role of GPR30 in the PyMT$^{+/+}$ mouse model of breast carcinogenesis.

Mammary tumors from GPR30$^{-/-}$PyMT and GPR30$^{+/+}$PyMT$^{+/+}$ mice were harvested when the mice were 13 weeks old. Tumors were weighed to determine tumor size and paraffin embedded to analyze the receptor status and grade of the tumors. Although there was no difference in latency, GPR30$^{-/-}$PyMT$^{+/+}$ mice have significantly smaller tumors than GPR30$^{+/+}$PyMT$^{+/+}$ mice. This suggests that GPR30 promotes breast carcinogenesis in this mouse model of breast cancer. Sections from the 5 largest tumors from 5 individual mice within each group were H&E stained and analyzed by a veterinary pathologist. The pathologist reported tumor grade as well as mitotic index, which is a rough estimation of proliferation. GPR30$^{-/-}$PyMT$^{+/+}$ mice show a trend to have lower grade tumors and a lower mitotic index consistent with GPR30 mediating breast carcinogenesis.

Because GPR30 knockout mice had smaller, lower grade tumors, we wanted to further analyze the effects of GPR30 by specifically targeting it with the GPR30-selective agonist, G-1 and antagonist, Gantag. PyMT$^{+/+}$ mice were ovariectomized at 3 weeks of age to remove most of the endogenous estrogen. The mice were allowed to recover for 7 days and then a 60 day release pellet was implanted. The pellet treatments included sham, E2, G-1, Gantag, and E2 + Gantag, to determine the contribution of GPR30 to E2-dependent growth. The tumors were harvested at 10, 12, or 14 weeks and weighed to determine if there is any difference in tumor size between treatments. As expected, E2 increased tumor growth significantly at all time points. However, GPRantag treatment alone and in combination with E2 had no effect on tumor size. Surprisingly, the G-1 treated mice had smaller tumors than sham at 14 weeks, while there were no significant differences at earlier time points. Sections of the 5 largest tumors from individual mice at 12 weeks old were H&E stained and analyzed by the veterinary pathologist. There was a trend for G-1 treated tumors to be lower grade as well as have a lower mitotic index compared to sham, while all other treatments looked the same as sham. These data suggest that stimulating GPR30 with G-1 hinders the progression of mammary tumors.

Aim 3: The third aim of this study proposes to look at metastasis in PyMT$^{+/+}$ mice with respect to GPR30.

The tumors from PyMT$^{+/+}$ mice metastasize to the lungs via the blood. Therefore, at the time the tumors were resected from the PyMT$^{+/+}$ mice, we also collected blood and lungs and stored them at -80°C. Metastasis was assessed by the presence of PyMT RNA in the lungs, since the lung should not express PyMT RNA unless metastasis from the tumors is present [17]. The lungs from 14 week-old mice were probed for metastasis and G-1 treated mice had no metastasis while 50-60% of mice treated with sham or Gantag had distant metastasis. These data suggest that stimulation of GPR30 with G-1 inhibits the ability of the breast tumor cells to metastasize to the lung.

Key Research Accomplishments
- GPR30 enhances the proliferation of breast cancer cells *in vitro*

- The absence of GPR30 in the PyMT$^{+/+}$ model of breast carcinogenesis does not affect the latency of the tumor growth
- GPR30$^{-/-}$PyMT$^{+/+}$ mice have smaller tumors than GPR30$^{+/+}$PyMT$^{+/+}$ mice
- There is a trend for GPR30$^{-/-}$PyMT$^{+/+}$mice to have lower grade tumors with a lower mitotic index compared to GPR30$^{+/+}$PyMT$^{+/+}$ mice
- Targeting GPR30 with G-1, the GPR30-selective agonist, in a PyMT$^{+/+}$ mouse decreases tumor size compared to sham treated mice without affecting latency
- G-1 treated PyMT+/+ mice trend to have a lower grade and mitotic index compared to sham treated mice
- Treatment of PyMT$^{+/+}$ mice with G-1 inhibited distant metastasis to the lungs

Reportable Outcomes
- This work was presented at the 25th Annual National MD/PhD Conference

Conclusions

Using the SUM149 cell line as a model of human breast cancer and the PyMT cell line as a model of mouse mammary cancer, we were able to determine that GPR30 stimulation increases cell number in a crystal violet assay, suggesting an increase in proliferation. To further understand the role of GPR30 in a complex tumor environment, we employed the PyMT$^{+/+}$ mouse model of breast carcinogenesis. GPR30$^{-/-}$PyMT$^{+/+}$ mice had smaller tumors, which exhibited a trend to have a lower grade and mitotic index. These data are consistent with GPR30 playing a growth-promoting role in breast cancer. However, treatment of PyMT$^{+/+}$ mice with the GPR30-selective agonist, G-1, hindered tumor growth. The G-1 treated tumors also trended to have a lower tumor grade and mitotic index. Finally G-1 treatment inhibited distant metastasis to the lungs. On the surface these data seem contradictory, which means more research is needed to understand the function of GPR30 in breast carcinogenesis.

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